Postharvest Biology and Technology 49 (2008) 221–228

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Impact of storage temperature, storage duration, and harvest date on sugarbeet raffinose metabolism

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ARTICLE INFO

Article history:
Received 10 May 2007
Accepted 18 February 2008

Keywords:
Beta vulgaris L.
Galactinol synthase
α-Galactosidase
Postharvest storage
Raffinose synthase

ABSTRACT

Raffinose negatively impacts sugarbeet (*Beta vulgaris* L.) processing by decreasing extractable sucrose yield and altering sucrose crystal morphology which reduces filtration rates and slows processing. Although increased raffinose concentrations have been observed during cold storage, the physiological and biochemical mechanisms associated with raffinose accumulation in sugarbeet are poorly understood. The objective of this study was to characterize the impact of storage temperature, storage duration, and harvest date on raffinose metabolism. Field-grown sugarbeets were harvested 7 September, 27 September, and 26 October 2004, and stored for 2, 10, or 18 weeks at 2 °C or 6 °C. Raffinose concentrations were approximately double at 2 weeks of storage, nearly threefold higher at 10 weeks, and decreased slightly at 18 weeks. Delaying harvest date increased raffinose concentration at harvest (0 weeks), but decreased concentrations at 18 weeks of storage. Storage temperature did not affect crown raffinose concentrations, but root tissues stored at 2 °C had 19% higher raffinose concentrations than at 6 °C. Biosynthetic or catabolic enzyme activities accounted for less than 15% of the variation in raffinose content in storage, although a small positive correlation ($r = 0.28$) between raffinose synthase activity and raffinose concentration in root tissues was observed. Galactinol synthase was highly expressed in tissues collected in late October and at 2 weeks of storage, and α-galactosidase activity increased 55% in roots stored for 18 weeks at 6 °C. Factors contributing to sugarbeet raffinose accumulation in storage are complex as raffinose concentrations were impacted by storage duration, harvest date, and storage temperature.

1. Introduction

Sucrose loss during sugarbeet storage results from sucrose biochemical transformations into non-sucrose impurities including the trisaccharide, raffinose. Raffinose (*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 ↔ 2)-*O*-β-D-fructofuranoside) is a naturally occurring sugar found in sugarbeet that hinders sugarbeet processing. Raffinose is typically present at 0.3–0.5% of the total sucrose content but concentrations may increase two to fivefold during storage (McGinnis, 1951; Wyse and Dexter, 1971; Martin et al., 2001). Transformation of sucrose to raffinose increases factory operation costs and reduces extractable sucrose yields (McGinnis, 1951). Elevated raffinose concentrations decrease the rate of sucrose crystallization by as much as 50%, lead to production of elongated sucrose crystals that hinder separation of sucrose crystals from molasses, and increase the loss of sucrose to molasses (McGinnis, 1951).

Several studies have shown an association between low temperature and raffinose accumulation in sugarbeet (Walker et al., 1960; McCready and Goodwin, 1966; Wyse and Dexter, 1971). In a study conducted by Walker et al. (1960), sugarbeet raffinose concentrations were approximately 2.5-fold higher after 60 d of storage at 1 °C than at 12 °C. McCready and Goodwin (1966) also reported an increase in raffinose concentration after low temperature storage (2 °C), but found that raffinose concentration could be reduced to near harvest levels by storage at 25 °C for 25 d. Harvest date also is reported to influence sugarbeet root raffinose concentration at harvest and after storage. Finkner et al. (1959) found that roots harvested in mid-September had 34% lower raffinose concentration at harvest than roots harvested in early December. Similarly, Wyse and Dexter (1971) reported that sugarbeet harvested in September and early October had lower raffinose contents when compared to beets harvested in November but earlier harvested beets had greater accumulation of raffinose after 65 d in storage.

Raffinose is not equally distributed throughout the taproot, and raffinose concentrations are twofold greater in the sugarbeet crown than in the true root (Jaggard et al., 1999). The increased raffinose content in sugarbeet crown tissue may be attributed to greater exposure of this tissue to low temperatures prior to harvest. Raffinose is believed to help stabilize membrane structure during

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doi:10.1016/j.postharvbio.2008.02.007

periods of low temperature (Santarius, 1973), and its accumulation is closely associated with cold acclimation and improved cold hardiness in several herbaceous plants including common bugle (*Ajuga reptans*) and alfalfa (*Medicago sativa* L.) (Bachmann et al., 1994; Castonguay et al., 1995; Cunningham et al., 2003).

Raffinose synthesis is catalyzed by raffinose synthase (RS; EC 2.4.1.82), an enzyme that transfers a galactosyl unit from galactinol to sucrose (Lehle and Tanner, 1973). The synthesis of galactinol is catalyzed by galactinol synthase (GS; EC 2.4.1.123), a transferase that catalyzes the condensation of *myo*-inositol with UDP-galactose. It has been suggested that GS has a role in determining raffinose content in some plant species. Saravitz et al. (1987) evaluated carbohydrate partitioning among soybean (*Glycine max*) genotypes with contrasting raffinose concentrations, and found a linear relationship between GS activity and raffinose. Castillo et al. (1990) reported that GS activity is cold inducible in leaves and seeds of soybean and kidney bean (*Phaseolus vulgaris*). Raffinose is degraded by α-galactosidase (α-GAL; EC 3.2.1.22), which catalyzes the hydrolytic cleavage of the terminal-linked galactose moiety (Dey, 1981).

Although increased raffinose concentrations have been found in sugarbeet tissues typically stored at 4 °C or below, the molecular and biochemical factors contributing to sugarbeet raffinose biosynthesis and catabolism are largely unknown. The objective of this research was to investigate the biochemical mechanisms regulating sugarbeet raffinose accumulation during postharvest storage. The impact of storage temperature, storage duration, and harvest date on raffinose concentrations, raffinose biosynthetic gene expression, and enzyme activities involved in raffinose metabolism were characterized from crown and root tissues. Field grown sugarbeets were harvested on three dates: 7 September, 27 September, and 26 October 2004, and tissues were analyzed at harvest or after 2, 10 or 18 weeks of storage at 2 and 6 °C. Since raffinose concentration differs between the root crown and the true root (Jaggard et al., 1999), crown and root tissues were analyzed separately.

2. Materials and methods

2.1. Plant material

Sugarbeet hybrid, ACH192 (American Crystal Sugar Company, Moorhead, MN) were produced in Fargo, ND. Seedlings were established 18 May 2004, and fertilization and weed control were in accordance with recommended production guidelines. Roots were hand harvested 7 September, 27 September, and 26 October 2004, and only healthy, regularly shaped beets were sampled. There were four replicates for each treatment and each replicate consisted of a polyethylene bag containing four sugarbeets. Immediately after harvest, roots were hand washed, placed in perforated polyethylene bags, and stored at 95% relative humidity at 2 or 6 °C. Sugarbeet tissues were collected at harvest and after 2, 10, and 18 weeks of storage. At each sampling, crown and root tissues were collected by separating roots at the lowest leaf scar. Tissues were diced into small pieces, immersed in liquid N_2, lyophilized, ground to a fine powder, and stored at −80 °C. The experiment was repeated in successive years with similar results.

2.2. Raffinose quantification

Crown and root raffinose concentrations were determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) using lactose as an internal standard as reported previously (Klotz and Finger, 2002). Raffinose was eluted isocratically with 200 mmol L^{-1} NaOH at a flow rate of 0.017 mL s^{-1} using a 4 mm × 250 mm CarboPac PA10 column (Dionex Corp., Sunnyvale, CA) equipped with a 4 mm × 50 mm CarboPac PA-10 guard column. Peak identity and raffinose concentration were determined by comparison with raffinose standards (Sigma–Aldrich, St. Louis, MO).

2.3. Protein extraction and enzyme activity assays

Protein analyses were conducted at 4 °C unless otherwise stated. Proteins were extracted as previously described (Klotz and Finger, 2004). Crown or root tissues were homogenized in 10 volumes (w/v) of extraction buffer (100 mmol L^{-1} Hepes–NaOH, pH 7.2, 10 mmol L^{-1} Na_2SO_3, 5 mmol L^{-1} 1,4-dithiothreitol [DTT], 1 mmol L^{-1} $MgCl_2$), and the homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA). The filtrate was centrifuged at 12,000 × *g* for 30 min. The supernatant was dialyzed against a solution of 10 mmol L^{-1} Hepes–NaOH, pH 7.2, 1 mmol L^{-1} DTT, and 1 mmol L^{-1} $MgCl_2$, and the desalted supernatant was subsequently used for GS, RS, and α-GAL activity assays. Total protein was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

2.3.1. Galactinol synthase activity

Galactinol synthase activity was determined by a modification of the method of Liu et al. (1995). Dialyzed protein extracts (22 μL) were assayed for GS activity in duplicate 50 μL reactions containing 50 mmol L^{-1} Hepes–NaOH, pH 7.2; 4 mmol L^{-1} $MnCl_2$; 2 mmol L^{-1} DTT; 4 mmol L^{-1} [^{3}H]-UDP-galactose (Gal-6-^{3}H, 4.7 Ci mmol^{-1}; Sigma–Aldrich, St. Louis, MO). Reactions were initiated by the addition of 20 mmol L^{-1} *myo*-inositol. Duplicate tubes in which water replaced *myo*-inositol served as blanks. Assays were run for 30 min at 30 °C and terminated by adding 200 μL of 100% ethanol. Unreacted UDP-galactose was removed by adding 300 μL of a 1:1 (v/w) slurry of water:Dowex-1 (formate form; Sigma–Aldrich, St. Louis, MO). Tubes were vortexed and centrifuged at 10,000 × *g* for 10 min. After centrifugation, 100 μL supernatant was added to 5 mL Bio-Safe II scintillation cocktail (Research Products International, Mount Prospect, IL) and samples were counted in a LS6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA).

2.3.2. Raffinose synthase activity

Raffinose synthase activity was determined by a modification of the method of Bachmann et al. (1994). Dialyzed protein extracts (45 μL) were assayed for RS activity in duplicate 50 μL reactions containing 5 mmol L^{-1} galactinol (Wako BioProducts, Richmond, VA) and 40 mmol L^{-1} sucrose. Duplicate microfuge tubes in which water replaced galactinol served as blanks. Reactions proceeded for 30 min at 30 °C, and were terminated by boiling in a water bath for 5 min. Samples were diluted 10-fold with water, centrifuged for 10 min at 14,000 × *g*, and the supernatant was filtered through a 0.22 μm filter. Raffinose was quantified with HPAE-PAD as described previously.

2.3.3. α-Galactosidase activity

α-Galactosidase activity was determined by a modification of the method of Kuo et al. (1997). Dialyzed extracts (80 μL) were assayed for α-GAL activity in triplicate. Assay mixtures contained protein extract in 70 mmol L^{-1} MES buffer (pH 6.0), and the reaction was initiated with 5 mmol L^{-1} *p*-nitrophenyl α-D-galactopyranoside (Sigma–Aldrich, St. Louis, MO). After 30 min at 30 °C, the reaction was terminated by the addition of 120 μL 1.0 mol L^{-1} Na_2CO_3. Blanks were prepared by adding enzyme, buffer, and substrate after the addition of Na_2CO_3. The release of product was measured spectrophotometrically at 410 nm using a

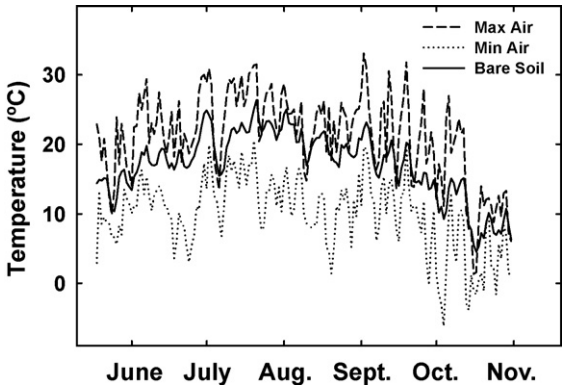

Fig. 1. Daily maximum and minimum air temperature and bare soil temperature at the Fargo, ND experiment station in 2004.

SpectraMax microplate reader (Molecular Devices Corp., Sunnyvale, CA). The amount of *p*-nitrophenol released was calculated using the molar extinction coefficient of 1.83×10^6 L mol^{-1} m^{-1}.

2.4. RNA isolation and RNA blot analysis

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA was glyoxylated, fractionated on a 1.0% agarose gel and transferred to a Hybond N$^+$ positively charged membrane (Amersham Bioscience Corp, Piscataway, NJ). RNA loading levels were assessed by staining membranes with methylene blue (Molecular Research Center, Inc., Cincinnati, OH). For RNA hybridization analysis, an 1178 bp fragment (875–2053 bp) from a sugarbeet raffinose synthase gene (GenBank Accession E37133), and a sugarbeet galactinol synthase EST (GenBank Accession BQ591564) were labeled with [^{32}P]-dCTP using the RadPrime labeling system (Invitrogen, Carlsbad, CA). Hybridization and washing conditions are described in Gana et al. (1997).

2.5. Statistical analysis

Data were analyzed using analysis of variance PROC ANOVA (SAS Institute, 2003). An F-protected L.S.D. ($P \leq 0.05$) was calculated for comparison of main effect means and two-way interactions. Pearson correlation coefficients (r) for raffinose concentration and individual enzyme activities were calculated using SigmaStat for Windows, version 2.03 (SPSS Inc., Chicago, IL).

3. Results

Field grown sugarbeets were harvested on three dates: 7 September, 27 September, and 26 October 2004, and tissues were collected for laboratory analyses at harvest and after 2, 10 or 18 weeks of storage at 2 or 6 °C. Maximum and minimum air temperatures and bare soil temperature during the 2004 growing season at the Fargo experiment station are reported in Fig. 1. The average minimum air temperature for the weeks of 7 September, 27 September, and 26 October was 12, 7, and 4 °C; the bare soil temperature was 17, 15, and 9 °C, respectively.

3.1. Impact of harvest date, storage duration, and storage temperature on raffinose concentrations

3.1.1. Crown

Storage temperature did not significantly affect crown raffinose concentrations (Table 1). Crown raffinose concentrations were affected by storage duration, and the interaction of harvest date with storage duration. Crown raffinose concentrations were approximately double at 2 weeks of storage (6.7 g kg^{-1}) and were approximately threefold higher at 10 weeks (9.9 g kg^{-1}) compared to initial harvest concentrations (3.2 g kg^{-1}). Crown raffinose concentrations decreased between 10 and 18 weeks of storage, but were still 44% higher than initial concentrations. When averaged across storage temperature and duration, harvest date had no significant impact on overall crown raffinose concentrations. However, delaying the harvest date had a significant impact on initial raffinose concentrations and concentrations at 18 weeks of storage. Raffinose concentrations at harvest were lowest from sugarbeet harvested 7 September (1.2 g kg^{-1}), highest from sugarbeets harvested 26 October (5.4 g kg^{-1}), with intermediate crown raffinose concentrations (3.0 g kg^{-1}) from the 27 September harvest. In contrast, delayed harvest date was associated with decreased raffinose concentration after 18 weeks of storage. At 18 weeks of storage, crown raffinose concentrations were highest from sugarbeet harvested 7 September (6.5 g kg^{-1}), lowest from crown tissues harvested 26 October (2.3 g kg^{-1}), and intermediate from sugarbeets harvested 23 September (5.2 g kg^{-1}).

Crown raffinose concentrations were also affected by the interaction of storage temperature and harvest date. Temperature impacted crown raffinose concentrations in sugarbeet harvested 7 September and 26 October, but not from crown tissues harvested 27 September. Sugarbeet harvested 7 September had less raffinose when stored at 2 °C (5.7 g kg^{-1}) than at 6 °C (6.9 g kg^{-1}). In contrast, crown tissues from sugarbeet harvested 26 October were greater at 2 °C (6.6 g kg^{-1}) than from 6 °C (5.5 g kg^{-1}).

3.1.2. Root

Root raffinose concentrations were affected by storage temperature, storage duration and the interaction of harvest date with storage duration (Table 1). Roots stored at 2 °C had 19% more raffinose than roots stored at 6 °C. Root raffinose concentrations were approximately double at 2 weeks of storage (4.0 g kg^{-1}) and were nearly threefold higher at 10 weeks (6.1 g kg^{-1}) when compared to initial harvest concentrations (2.2 g kg^{-1}). Root raffinose concentrations decreased between 10 and 18 weeks of storage, but were still 55% higher than initial concentrations. Delaying harvest date had a significant impact on initial raffinose concentrations and concentrations at 18 weeks of storage. Raffinose concentrations at harvest were lowest from root tissues harvested 7 September (0.8 g kg^{-1}), highest from roots harvested 26 October (3.6 g kg^{-1}), and intermediate in roots (2.2 g kg^{-1}) from the 27 September harvest. In contrast, delayed harvest date was associated with decreased raffinose concentration after 18 weeks of storage. At 18 weeks of storage, root raffinose concentrations were highest from sugarbeet root tissues harvested 7 September (5.0 g kg^{-1}), lowest from roots harvested 26 October (1.8 g kg^{-1}), and intermediate from root tissues harvested 23 September (3.4 g kg^{-1}).

Root raffinose concentrations were also affected by the interaction of storage temperature and duration. There was no impact of storage temperature on raffinose concentration from roots at 0, 2, or 18 weeks of storage, but roots stored for 10 weeks at 2 °C had 44% higher raffinose concentration than roots stored at 6 °C for 10 weeks.

3.2. Raffinose biosynthetic gene expression during sugarbeet storage

3.2.1. Crown mRNA levels

Harvest date, storage duration, and storage temperature influenced crown GS and RS steady-state mRNA levels (Fig. 2A). Delaying the harvest date increased initial (week = 0) crown GS gene expression. Galactinol synthase transcripts were low

Table 1
Influence of storage temperature (*T*), storage duration (*D*), and harvest date (*H*) on crown and root raffinose concentrations

Duration (weeks)	7 September harvest			27 September harvest			26 October harvest			Temperature		Duration
	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean
Crown raffinose concentrations (g kg^{-1})												
0	1.1	1.3	1.2	2.8	3.2	3.0	5.3	5.5	5.4	3.1	3.3	3.2 *d*
2	4.2	7.0	5.6	5.6	7.0	6.3	8.9	7.5	8.2	6.2	7.1	6.7 *b*
10	12.1	11.9	12.0	9.6	9.3	9.4	10.0	6.8	8.4	10.6	9.3	9.9 *a*
18	5.5	7.5	6.5	5.6	4.8	5.2	2.4	2.2	2.3	4.5	4.8	4.6 *c*
Mean	5.7	6.9	6.3 a	5.9	6.1	6.0 a	6.6	5.5	6.1 a	6.1 **a**	6.2 **a**	
Root raffinose concentrations (g kg^{-1})												
0	1.0	0.6	0.8	2.0	2.3	2.2	3.8	3.4	3.6	2.3	2.1	2.2 *d*
2	3.4	4.1	3.8	3.7	3.1	3.4	5.3	4.3	4.8	4.1	3.8	4.0 *b*
10	8.1	6.1	7.1	7.5	5.2	6.4	6.0	3.8	4.9	7.2	5.0	6.1 *a*
18	5.5	4.5	5.0	3.3	3.5	3.4	1.5	2.2	1.8	3.4	3.4	3.4 *c*
Mean	4.5	3.8	4.2 a	4.1	3.5	3.8 a	4.1	3.4	3.8 a	4.3 **a**	3.6 **b**	

Raffinose concentration is expressed as g kg^{-1} dry weight. For storage temperature, duration, and harvest date main effects, means followed by the same letter are not significantly different at $P \leq 0.05$. Letters to denote significance are in bold for storage temperature means, italicized for storage duration means, and in normal font for harvest date means. The least significant difference L.S.D. ($P \leq 0.05$) for two-way and higher order interaction mean comparisons for crown and root tissues are as follows: L.S.D.$_{(crown)}$ $T \times D$ = NS; $H \times D$ = 1.5; $T \times H$ = 1.1; $T \times D \times H$ = NS. L.S.D.$_{(root)}$ $T \times D$ = 0.8; $H \times D$ = 1.0; $T \times H$ = NS; $T \times D \times H$ = NS.

from crown tissues harvested 7 September, increased in tissues harvested 27 September, and greater in crowns harvested 26 October. Crown RS steady-state mRNA levels were also affected by harvest date (Fig. 2A). When comparing crown RS mRNA levels among the three harvest dates (week = 0), tissues harvested 7 September and 27 September had greater transcript abundance when compared to 26 October levels. During storage, crown GS and RS transcript levels were highest at 2 weeks, decreased at 10 weeks, and were barely detectable by 18 weeks. Temperature also affected crown raffinose biosynthetic gene expression as transcript levels for GS and RS were generally greater in crown tissues stored at 2 °C than those stored at 6 °C.

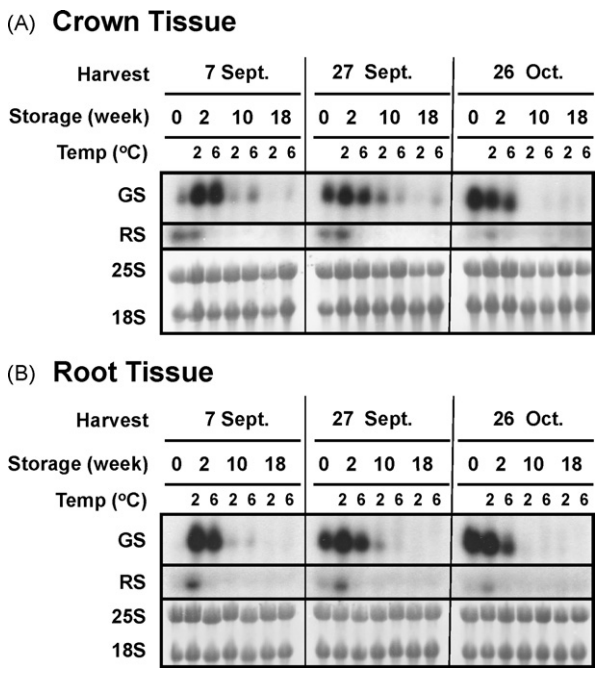

Fig. 2. Crown (A) and root (B) raffinose biosynthetic gene expression during sugarbeet storage. Northern hybridization (10 μg total RNA per lane) with ^{32}P-labeled galactinol synthase (GS) and raffinose synthase (RS) probes. Field-grown sugarbeets were harvested 7 September, 27 September, and 26 October 2004, and stored for 0, 2, 10, and 18 weeks at 2 or 6 °C. Lower panel is a methylene blue stained membrane showing 18 and 25 S ribosomal RNA levels.

3.2.2. Root mRNA levels

Harvest date, storage duration, and storage temperature influenced root raffinose biosynthetic gene expression (Fig. 2B). Delaying the harvest date increased initial (week = 0) root GS mRNA levels. Galactinol synthase transcripts were not detected from roots harvested 7 September, were present in root tissues harvested 27 September, and were present at their greatest levels in roots harvested 26 October. During storage, GS transcript levels were highest at 2 weeks, greatly decreased at 10 weeks, and were not detected by 18 weeks. At 2 weeks of storage, root tissues stored at 2 °C generally had higher GS transcript levels than roots stored at 6 °C.

Raffinose synthase steady-state mRNA levels from root tissues were affected by storage temperature and duration, but not by harvest date (Fig. 2B). Root RS transcript levels were highest at 2 weeks of storage, and transcript levels were below detection at 10 and 18 weeks of storage. When compared with tissue from roots stored at 6 °C, sugarbeet root tissues stored for 2 weeks at 2 °C had increased RS transcript levels.

3.3. Influence of harvest date, storage duration, and storage temperature on galactinol synthase enzyme activity

3.3.1. Crown

Crown GS activity was affected by storage duration and harvest date, but not storage temperature (Table 2). Activity was highest at 2 weeks (36.6 μmol kg^{-1} s^{-1}), decreased sevenfold at 10 weeks to 5.3 μmol kg^{-1} s^{-1}, and further decreased to 1.4 μmol kg^{-1} s^{-1} at 18 weeks. Delaying harvest date significantly increased initial crown GS activity levels. Initial activity (week = 0) from crown tissues harvested 26 October was on average 10-fold greater than from crown tissues harvested in September. However, at 2 weeks of storage, activity in crown tissues harvested 26 October was less than that in crown tissues harvested in September. Galactinol synthase activity in crown tissues was highest in sugarbeet harvested 7 September (47.1 μmol kg^{-1} s^{-1}), intermediate in sugarbeet harvested 27 September (38.7 μmol kg^{-1} s^{-1}), and lowest in sugarbeet harvested 26 October (23.9 μmol kg^{-1} s^{-1}) at 2 weeks of storage. Enzyme activity at 10 and 18 weeks of storage was similar among the three harvest dates. The interaction of storage temperature and harvest date was also significant. Storage temperature did not impact crown enzyme activity from tissues harvested 7 September or 27 September but crown tissues harvested 26 October had a 50% increase in enzyme activity when tissues were stored at 2 °C.

Table 2
Influence of storage temperature (*T*), storage duration (*D*), and harvest date (*H*) on crown and root galactinol synthase activity

Duration (weeks)	7 September harvest			27 September harvest			26 October harvest			Temperature		Duration
	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean
Crown galactinol synthase activity (μmol kg^{-1} s^{-1})												
0	3.8	5.0	4.4	1.8	3.0	2.4	34.2	32.3	33.2	13.3	13.4	13.4 *b*
2	44.0	50.3	47.1	35.4	41.9	38.7	33.7	14.1	23.9	37.7	35.4	36.6 *a*
10	10.5	3.4	6.9	3.0	7.8	5.4	6.4	0.7	3.6	6.6	4.0	5.3 *c*
18	0.0	3.4	1.5	1.2	0.2	0.7	0.4	3.3	1.8	0.5	2.3	1.4 *d*
Mean	14.5	15.5	15.0 a	10.4	13.2	11.8 b	18.7	12.6	15.6 a	14.5 **a**	13.8 **a**	
Root galactinol synthase activity (μmol kg^{-1} s^{-1})												
0	1.7	2.0	1.9	20.5	21.6	21.0	30.4	35.4	32.9	17.5	19.7	18.6 *b*
2	83.0	32.7	57.9	64.0	31.8	47.9	67.4	20.3	43.9	71.5	28.3	49.9 *a*
10	2.9	1.0	1.9	3.0	5.3	4.1	2.5	1.3	1.9	2.8	2.5	2.6 *c*
18	1.7	3.4	2.5	0.7	1.0	0.9	0.2	1.0	0.6	0.1	1.8	1.3 *c*
Mean	22.3	9.8	16.1 a	22.0	14.9	18.5 a	25.1	14.5	19.8 a	23.2 **a**	13.1 **b**	

Galactinol synthase is expressed as μmol kg^{-1} protein s^{-1}. For storage temperature, duration, and harvest date main effects, means followed by the same letter are not significantly different at $P \leq 0.05$. Letters to denote significance are in bold for storage temperature means, italicized for storage duration means, and in normal font for harvest date means. The least significant difference L.S.D. ($P \leq 0.05$) for two-way and higher order interaction mean comparisons for crown and root tissues are as follows: L.S.D.$_{(crown)}$ $T \times D$ = NS; $H \times D$ = 4.9; $T \times H$ = 3.5; $T \times D \times H$ = 7.0. L.S.D.$_{(root)}$ $T \times D$ = 5.4; $H \times D$ = 6.6; $T \times H$ = NS; $T \times D \times H$ = NS.

3.3.2. Root

Root GS activity was influenced by storage duration and temperature, but not harvest date (Table 2). Storing sugarbeet at 2 °C increased root GS activity nearly twofold compared to roots stored at 6 °C. Galactinol synthase activity was highest after 2 weeks storage (49.9 μmol kg^{-1} s^{-1}), decreased 19-fold after 10 weeks of storage (2.6 μmol kg^{-1} s^{-1}), and remained low after 18 weeks storage (1.3 μmol kg^{-1} s^{-1}). Root galactinol synthase was also affected by the interaction of storage temperature and duration. Roots stored for 2 weeks at 2 °C (71.5 μmol kg^{-1} s^{-1}) were 2.5-fold greater in activity than roots stored for 2 weeks at 6 °C (28.3 μmol kg^{-1} s^{-1}) but storage temperature had no impact on root GS activity at 0, 10, or 18 weeks of storage.

Delaying the harvest date significantly increased initial root GS activity. Enzyme activity from roots harvested 26 October (32.9 μmol kg^{-1} s^{-1}) was 17.5-fold greater than that from the 7 September harvest (1.9 μmol kg^{-1} s^{-1}) and was 57% greater than activity at the 27 September harvest (21.0 μmol kg^{-1} s^{-1}). At 2 weeks of storage, however, the activity from roots harvested 7 September was on average 26% greater than the activity from roots harvested 27 September and 26 October. Enzyme activity at 10 and 18 weeks of storage was similar among the three harvest dates.

3.4. Influence of harvest date, storage duration, and storage temperature on raffinose synthase enzyme activity

3.4.1. Crown

Storage duration and temperature affected crown RS activity (Table 3). Averaged across all harvests, activity at 2 weeks of storage (5.0 μmol kg^{-1} s^{-1}) was nearly 31% higher than the initial activity (3.8 μmol kg^{-1} s^{-1}). Activity at 10 weeks (3.9 μmol kg^{-1} s^{-1}) was similar to initial levels (week 0), but at 18 weeks activity was decreased by approximately 30% (2.7 μmol kg^{-1} s^{-1}). Enzyme activity was 58% higher in crown tissues stored at 2 °C (4.7 μmol kg^{-1} s^{-1}) than at 6 °C (3.0 μmol kg^{-1} s^{-1}). The interaction of storage duration and temperature was also significant. At 2 °C, RS activity increased 63% at 2 weeks of storage, remained elevated at 10 weeks, and at 18 weeks declined to levels found at harvest. In

Table 3
Influence of storage temperature (*T*), storage duration (*D*), and harvest date (*H*) on crown and root raffinose synthase activity

Duration (weeks)	7 September harvest			27 September harvest			26 October harvest			Temperature		Duration
	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean
Crown raffinose synthase activity (μmol kg^{-1} s^{-1})												
0	5.5	5.1	5.3	2.2	2.5	2.3	3.6	3.7	3.7	3.8	3.8	3.8 *b*
2	7.9	6.3	7.1	4.8	2.3	3.5	5.7	2.7	4.2	6.1	3.8	5.0 *a*
10	5.5	2.4	3.9	5.8	2.4	4.1	4.9	2.5	3.7	5.4	2.4	3.9 *b*
18	5.1	1.6	3.3	3.1	0.6	1.8	2.5	3.4	2.9	3.5	1.9	2.7 *c*
Mean	6.0	3.8	4.9 a	4.0	1.9	3.0 c	4.2	3.1	3.6 b	4.7 **a**	3.0 **b**	
Root raffinose synthase activity (μmol kg^{-1} s^{-1})												
0	3.2	3.9	3.5	1.1	1.2	1.1	1.6	1.5	1.5	1.9	2.2	2.1 *c*
2	8.8	5.2	7.0	7.8	2.8	5.3	5.0	3.4	4.2	7.2	3.8	5.5 *a*
10	7.4	2.0	4.7	8.5	2.6	5.6	2.4	1.0	1.7	6.1	1.9	4.0 *b*
18	6.1	3.3	4.7	4.3	3.5	3.9	2.4	1.6	2.0	4.2	2.8	3.5 *b*
Mean	6.4	3.6	5.0 a	5.4	2.5	4.0 b	2.8	1.9	2.3 c	4.9 **a**	2.7 **b**	

Raffinose synthase is expressed as μmol kg^{-1} protein s^{-1}. For storage temperature, duration, and harvest date main effects, means followed by the same letter are not significantly different at $P \leq 0.05$. Letters to denote significance are in bold for storage temperature means, italicized for storage duration means, and in normal font for harvest date means. The least significant difference L.S.D. ($P \leq 0.05$) for two-way and higher order interaction mean comparisons for crown and root tissues are as follows: L.S.D.$_{(crown)}$ $T \times D$ = 1.3; $H \times D$ = 1.6; $T \times H$ = NS; $T \times D \times H$ = NS. L.S.D.$_{(root)}$ $T \times D$ = 1.6; $H \times D$ = NS; $T \times H$ = NS; $T \times D \times H$ = NS.

Table 4
Influence of storage temperature (*T*), storage duration (*D*), and harvest date (*H*) on crown and root galactosidase activity

Duration (weeks)	7 September harvest			27 September harvest			26 October harvest			Temperature		Duration
	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean	2 °C	6 °C	Mean
Crown galactosidase activity (μmol kg^{-1} s^{-1})												
0	35.1	35.0	35.1	30.0	31.4	30.7	27.6	28.8	28.2	30.9	31.7	31.3 *c*
2	34.4	35.3	34.9	40.9	36.5	38.7	32.1	33.3	32.7	35.8	35.0	35.4 *b*
10	48.7	43.2	46.0	35.9	50.1	43.0	35.6	44.2	39.9	40.1	45.9	43.0 *a*
18	30.8	53.2	42.0	43.5	52.2	47.8	29.5	42.5	36.0	34.6	49.3	42.0 *a*
Mean	37.3	41.7	39.5 a	37.6	42.5	40.0 a	31.2	37.2	34.2 b	35.3 **b**	40.5 **a**	
Root galactosidase activity (μmol kg^{-1} s^{-1})												
0	28.2	29.0	28.6	28.9	30.3	29.6	28.6	29.3	29.0	28.5	29.5	29.0 *b*
2	23.1	25.1	24.1	33.1	28.2	30.7	30.7	29.2	30.0	29.0	27.5	28.3 *b*
10	30.6	33.6	32.1	29.8	34.7	32.2	26.6	29.1	27.9	29.0	32.5	30.7 *b*
18	39.2	49.1	44.1	28.2	39.4	33.8	21.6	49.7	35.7	29.7	46.1	37.9 *a*
Mean	30.2	34.2	32.2 a	30.0	33.1	31.6 a	26.9	34.4	30.6 a	29.0 **b**	33.9 **a**	

Galactosidase activity is expressed as μmol kg^{-1} protein s^{-1}. For storage temperature, duration, and harvest date main effects, means followed by the same letter are not significantly different at $P \leq 0.05$. Letters to denote significance are in bold for storage temperature means, italicized for storage duration means, and in normal font for harvest date means. The least significant difference L.S.D. ($P \leq 0.05$) for two-way and higher order interaction mean comparisons for crown and root tissues are as follows: L.S.D.$_{(crown)}$ $T \times D = 5.8$; $H \times D$ = NS; $T \times H$ = NS; $T \times D \times H$ = NS. L.S.D.$_{(root)}$ $T \times D = 6.4$; $H \times D$ = NS; $T \times H$ = NS; $T \times D \times H$ = NS.

contrast, activity from crown tissues stored at 6 °C did not increase in storage, but decreased at 10 and 18 weeks of storage. Harvest date and the interaction of harvest date and storage duration affected crown RS activity. Compared with sugarbeet harvested 27 September and 26 October, sugarbeet harvested 7 September had increased enzyme activity at harvest and at 2 weeks of storage. There was, however, no impact of harvest date on crown raffinose synthase activity at 10 and 18 weeks of storage.

3.4.2. Root

Root raffinose synthase activity was affected by harvest date, storage duration, and temperature (Table 3). When averaged across storage duration and temperature, activity was generally highest from root tissues harvested in early September (5.0 μmol kg^{-1} s^{-1}) and decreased with delayed harvest (4.0 and 2.3 μmol kg^{-1} s^{-1} for 27 September and 26 October, respectively). Averaged across all harvests, activity increased approximately 2.7-fold at 2 weeks of storage, decreased 27% at 10 weeks and was similar, but approximately 70% greater than initial harvest levels (week 0), at 18 weeks. Root RS activity at 2 °C was nearly twice that from tissues stored at 6 °C. At 2 °C, RS activity increased 3.5-fold at 2 weeks of storage, remained threefold higher at 10 weeks, and decreased at 18 weeks, but remained twofold higher than the activity found at harvest. Activity from root tissues stored at 6 °C nearly doubled at 2 weeks, but decreased at 10 and 18 weeks to levels similar to those found at harvest.

3.5. Influence of harvest date, storage duration, and storage temperature on α-galactosidase enzyme activity

3.5.1. Crown

Crown α-GAL activity was affected by harvest date, storage duration, storage temperature, and the interaction of storage duration and temperature (Table 4). Galactosidase activity from crown tissues decreased with delayed harvest date. Crown activity was 16% higher from sugarbeet harvested 7 September or 27 September than from sugarbeet harvested 26 October. Crown tissues stored at 6 °C had 15% higher α-GAL activity than those stored at 2 °C. When compared with enzyme activity at harvest (31.3 μmol kg^{-1} s^{-1}), α-GAL activity was 13% higher at 2 weeks of storage (35.4 μmol kg^{-1} s^{-1}) and increased an additional 21% at 10 weeks (43.0 μmol kg^{-1} s^{-1}). At 18 weeks, activity from tissues stored at 6 °C remained elevated but activity from tissues stored at 2 °C decreased to levels found at harvest.

Table 5
Pearson correlation coefficients (*r*) for galactinol synthase (GS), raffinose synthase (RS), and α-galactosidase (α-GAL) enzyme activity and raffinose concentrations from crown and root tissues

Variable	Crown			Root		
	GS	RS	α-GAL	GS	RS	α-GAL
Raffinose	0.02	0.05	0.28***	−0.07	0.28***	0.04
GS		0.37***	−0.24*		0.34***	−0.21
RS			−0.33***			−0.11

*, ***Significant at $P < 0.05$ and 0.001 level of probability, respectively.

3.5.2. Root

Harvest date did not impact root α-GAL levels but storage duration, storage temperature, and the interaction of temperature and duration significantly affected root α-GAL activity (Table 4). Averaged across harvest dates, root α-GAL activity was similar at 0, 2, and 10 weeks of storage, and was approximately 28% higher at 18 weeks of storage. Throughout storage, α-GAL activity remained unchanged in roots stored at 2 °C, but activity increased 57% in roots stored for 18 weeks at 6 °C.

3.6. Correlations of raffinose concentrations with enzyme activities

The association between root and crown raffinose concentration and the activities of GS, RS, and α-GAL activity were determined (Table 5). Crown raffinose concentrations were positively associated with α-GAL activity, and not with GS or RS. Root raffinose concentrations were positively associated with RS activity, but were not significantly associated with GS or α-GAL activity.

4. Discussion

Previous research has shown that raffinose concentration increases during storage and increased concentration is primarily associated with storage temperatures below 4 °C (Walker et al., 1960; Wyse and Dexter, 1971; Martin et al., 2001). In this study, sugarbeet root tissues stored at 2 °C had raffinose concentrations that were 19% higher than those from 6 °C storage, although crown raffinose concentrations were unaffected by storage temperature.

Crown raffinose concentrations, however, were approximately 1.5-fold higher than root concentrations when averaged across all samples. The elevated raffinose concentration in crown tissues is in agreement with research conducted by Jaggard et al. (1999), who found approximately twofold greater raffinose concentration in crown than in root tissue. Low temperature exposure of crown, but not root tissues prior to harvest may explain this difference in raffinose concentration as cold-induced raffinose accumulation has been documented in several plant species (Bachmann et al., 1994; Castonguay et al., 1995; Taji et al., 2002). Storage at 2 °C may have induced raffinose accumulation in root tissues as the below ground root tissues were never subjected to temperatures less than 4 °C prior to storage (Fig. 1). Preharvest exposure of crown tissues to low temperatures, however, may have induced raffinose accumulation prior to harvest and prevented any additional induction of raffinose biosynthesis by cold storage temperatures.

Storage at 2 °C increased GS activity in the root but not the crown. Raffinose synthase activity, however, increased in both root and crown from sugarbeets stored at 2 °C relative to those stored at 6 °C. Crown and root tissues stored at 6 °C had approximately 15% higher α-GAL activity than those from 2 °C storage. Cold induction of GS gene expression and enzyme activity has previously been observed in soybean, kidney bean, *Arabidopsis*, *A. reptans*, and alfalfa (Castonguay and Nadeau, 1998; Liu et al., 1998; Castillo et al., 1990; Sprenger and Keller, 2000; Taji et al., 2002; Cunningham et al., 2003), while increased α-GAL gene expression and activity have been observed in petunia in response to increasing temperature during low temperature deacclimation (Pennycooke et al., 2004). An effect of temperature on RS activity in other plant species, however, has not, to our knowledge, been reported.

In addition to storage temperature, storage duration affected sugarbeet raffinose concentration. In northern sugarbeet production areas, roots are harvested in late autumn and stored up to 18 weeks prior to processing or freezing. Trends in raffinose accumulation throughout 18 weeks of storage were similar for both crown and root tissues. When compared to initial harvest concentrations, raffinose concentrations were approximately double at 2 weeks of storage and nearly threefold higher at 10 weeks and decreased between 10 and 18 weeks of storage. Galactinol synthase and raffinose synthase transcript abundance and enzyme activity were generally highest at 2 weeks of storage but declined significantly by 18 weeks. Although RS transcripts were undetectable after 10 and 18 weeks in storage, RS activity declined by only 46 and 36% in crown and root tissue, respectively, from their maximum values after 2 weeks storage suggesting that the enzyme, *in planta*, is relatively stable. Crown and root α-GAL activity increased 35% and 29% after 18 weeks of storage. The observed changes in raffinose biosynthetic and catabolic activities suggest that raffinose concentrations from sugarbeet at 18 weeks of storage may result from decreased raffinose biosynthesis and increased α-GAL enzyme activity. However, correlation analysis only revealed a positive relationship between root raffinose concentration and RS activity. Although decreased raffinose content has been observed concomitant with increased α-GAL activity in alfalfa and petunia (Castonguay and Nadeau, 1998; Pennycooke et al., 2004), no negative correlation between sugarbeet raffinose levels and α-GAL activity was observed.

To evaluate the impact of agronomic practices and additional external environmental factors on raffinose accumulation, field grown sugarbeets were harvested on three separate occasions representative of an early, typical, and late harvest. There was no impact of harvest date on overall raffinose concentrations when averaged across storage temperature and storage duration main effects. Harvest date, however, had a significant impact on initial raffinose concentrations and concentrations at 18 weeks of storage, and both root and crown raffinose concentrations were similarly affected by harvest date. At harvest, raffinose concentrations increased with each delay in harvest date. After 18 weeks of storage, however, raffinose concentrations were greater from early harvested tissues. The increased raffinose concentration with delayed harvest and the increased raffinose concentration in early harvested roots after prolonged storage are in agreement with earlier sugarbeet studies (Finkner et al., 1959; Wyse and Dexter, 1971). While low temperature induction of raffinose biosynthesis may explain the increase in crown raffinose concentration with delayed harvest, it does not explain the increase in root raffinose concentration as only the sugarbeet crown was exposed to cold preharvest temperatures. Similarly, temperature alone does not explain differences in raffinose concentration due to harvest date at 18 weeks storage. Harvest-related differences in raffinose concentrations after 18 weeks storage, moreover, cannot be easily explained by changes in raffinose biosynthetic or catabolic activities since GS and α-GAL activities in the root and crown and RS activity in the root were not significantly affected by harvest date at 18 weeks. Clearly, other factors that have yet to be identified influence raffinose accumulation during storage.

It has long been postulated that raffinose accumulation in sugarbeets during storage is primarily dependent on storage temperature (Walker et al., 1960; McCready and Goodwin, 1966; Wyse and Dexter, 1971). This study demonstrates that the factors contributing to sugarbeet raffinose accumulation in storage are much more complex as raffinose concentrations were significantly impacted by storage temperature, storage duration, and harvest date. This study also demonstrates that raffinose accumulation is not easily explained by changes in expression of raffinose biosynthetic genes or by changes in raffinose biosynthetic or catabolic enzyme activities. Steady-state transcription levels of raffinose biosynthetic genes were a poor predictor of raffinose concentrations and regression analysis of individual enzyme activities with raffinose concentrations throughout storage revealed that enzyme activity accounted for no more than 15% of the variation in raffinose content in root and crown tissues. Metabolite levels can be affected not only by gene transcription and enzyme activity but also by changes in the availability of substrates, the presence of enzyme activators or inhibitors, and cellular pH. These studies suggest that evaluation of these other factors may be required to understand raffinose accumulation in stored sugarbeets.

Acknowledgments

The authors thank John Eide and Michelle Howard for technical support and the Beet Sugar Development Foundation for financial support. The use of trade, firm, or corporation names is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

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